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An Essential Role for Daxx in the Inhibition of B Lymphopoiesis by Type I Interferons

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Summary

Interferon-α and -β inhibit the interleukin-7-mediated growth and survival of T and B lymphoid progenitors via an unknown, STAT1-independent pathway. Gene expression profile analysis of interferon-β-treated progenitor B cells revealed enhanced *Daxx* expression, with concomitant Daxx protein increase and nuclear body translocation. The interferon effects included downregulation of cell cycle regulating genes and cell cycle arrest, followed by Bcl-2 downregulation and apoptosis. Daxx antisense oligonucleotides rescued the interferon-treated pro-B cells from growth arrest and apoptosis in parallel with the reduction of nuclear Daxx. These findings implicate the gene repressor function of Daxx in interferon-induced apoptosis of lymphoid progenitors.

Introduction

A critical checkpoint in B lymphopoiesis occurs at the transition from the pro-B to pre-B cell stage in differentiation (Rajewsky, 1996). This progression requires the productive rearrangement of variable (V), diversity (D), and joining (J) region gene segments in the immunoglobulin heavy chain locus to create a functional variable region exon, thereby allowing expression of μ heavy chains together with the VpreB/λ5 surrogate light chain elements and the Igα/Igβ signaling components of the pre-B cell receptor (Karasuyama et al., 1990). B cell development is blocked at the pro-B cell level in mice and humans deficient in any one of the pre-B cell receptor components (reviewed by Conley and Cooper, 1998; Benschop and Cambier, 1999; Meffre et al., 2000). Pro-B cell differentiation is also blocked in mice deficient in either interleukin-7 (IL-7) or the IL-7 receptor (Namen et al., 1988; von Freeden-Jeffry et al., 1995; Peschon et al., 1994). B lymphopoiesis can also be regulated by exogenous inhibitory factors (Kincade, 1994), among which are the type I interferons, α and β (IFN-α and IFN-β). Type I interferons constitutively produced by bone marrow macrophages can reverse the lymphopoietic effects of IL-7 by inducing apoptosis of IL-7-responsive lymphoid progenitors (Wang et al., 1995). Treatment of mice with type I interferon effectively blocks B cell development at the pro-B cell stage (Lin et al., 1998), and it has been suggested that a physiological consequence of local type I interferon production may be the deletion of B cell progenitors that fail to undergo productive V(D)J gene segment rearrangement (Wang et al., 1995). Consistent with this prediction, mice lacking the type I interferon receptor have a skewed B cell repertoire and enhanced antibody production (Vasconcellos et al., 1999).

The mechanism by which type I interferons counteract the growth and survival signals elicited by IL-7 to induce apoptosis is unclear. Interferon-mediated signaling involves phosphorylation of tyrosine residues and activation of components in the Jak/Stat signaling pathway (Darnell, 1997; Haque and Williams, 1998; Liu et al., 1998; Platanias and Fish, 1999). The phosphorylated Stat proteins form complexes that migrate into the nucleus to bind promoter sequences of target genes to regulate their transcription (Qureshi et al., 1995; Darnell, 1997; Pellegrini and Dusanter-Fourt, 1997). STAT1 appears to be an essential component in this pathway, as mice lacking STAT1 are unresponsive to interferon-induced upregulation of a variety of target genes and inhibition of cell growth (Durbin et al., 1996; Meraz et al., 1996). Consequently, the STAT1-deficient mice are highly susceptible to viral infection and tumor progression, although they have no obvious abnormalities in lymphoid development. It was surprising, therefore, to find that STAT1 is not required for the type I interferon-induced apoptosis of B cell progenitors, thus providing evidence for an alternative pathway of interferon-mediated cell signaling (Gongora et al., 2000a). Analysis of the mechanism responsible for this mode of apoptosis in lymphoid progenitors is complicated by the apparent interaction between the IL-7 and interferon signaling pathways, which are known to share components of the Jak/Stat system (Foxwell et al., 1995; Darnell, 1997). Because a common consequence of interferon-mediated signaling is the regulation of gene transcription, we used DNA microarrays to identify alterations in the mRNA expression profile of an IL-7-dependent pro-B cell line in response to type I interferons. This analysis identifies *Daxx* as one of the genes whose expression is upregulated by type I interferons and shows it to be an essential regulatory element in this mode of lymphoid progenitor apoptosis.

Results

IFN-β-Induced Changes in the Gene Expression Profile of Pro-B Cells

The Scid7 cell line has a pro-B cell phenotype ($CD19^+CD43^+IgM^-$), grows in response to IL-7, and is sensitive to interferon-mediated apoptosis (Ogawa et al., 1989; Wang et al., 1995; Gongora et al., 2000a), thereby providing a clonal model for its polyclonal pro-B cell counterparts in the bone marrow. For the DNA microarray analysis, Scid7 cells were treated with IFN-β for 16 hr before RNA harvesting. The 16 hr time point was chosen to precede overt signs of interferon-induced apoptosis, which are evident by 24 hr (Wang et al., 1995; see Figure 1A). Before analysis of microarray data on the gene expression profiles of treated and untreated

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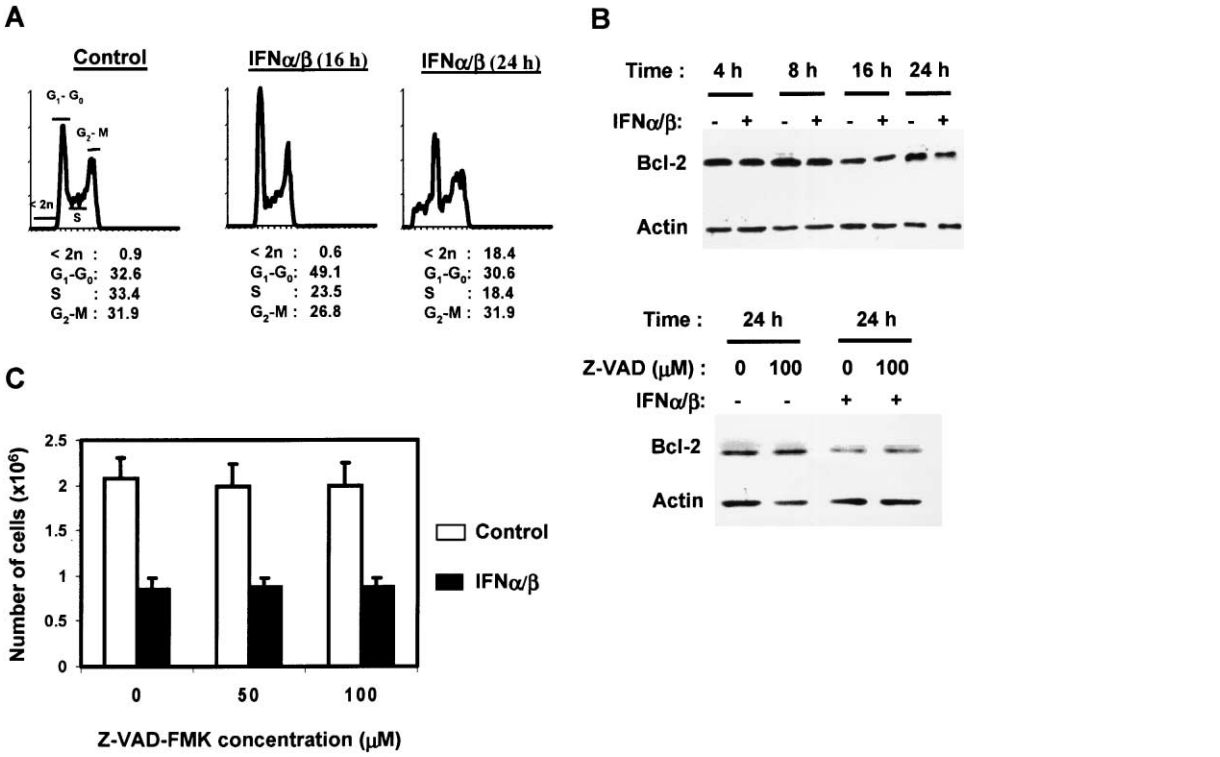


Figure 1. Type I Interferon Effects on Scid7 Pro-B Cell Cycle and Bcl-2 Expression

(A) The DNA content of Scid7 cells, untreated or treated for 16 hr with IFN-α/β, was analyzed by flow cytometry as described in Experimental Procedures.

(B) Cellular proteins from Scid7 cells, treated or untreated with IFN-α/β, were separated by SDS-PAGE before evaluation of Bcl-2 and actin levels by immunoblotting with specific antibodies. In some experiments, Scid7 cells were pretreated with the Z-VAD-FMK caspase inhibitor (100 μM) for 1 hr and then were treated or untreated with IFN-α/β for 24 hr.

(C) Scid7 cells were pretreated with Z-VAD-FMK at the indicated concentrations before IFN-α/β treatment for 24 hr and enumeration of viable cells in the presence of trypan blue.

Scid7 cells, the quality of synthesis reactions and hybridizations was confirmed (see Experimental Procedures). A comparative profile analysis of relative fluorescence intensities corresponding to gene expression before and after IFN-β treatment revealed 768 genes or expressed sequence tags (EST) that exhibited a >2-fold change in expression. Increased transcript levels were observed in the treated sample for 61% of these genes, whereas decreased transcription levels were observed for the remaining 39%. Genes found to have the greatest alteration in expression are listed in Table 1 (complete data sets available upon request). Many of the upregulated genes, particularly those showing the highest levels of enhancement, have been previously recognized as IFN-stimulated genes (ISGs). Transcripts for these genes were often undetectable in the untreated Scid7 cells (Table 1A). In contrast, a role for interferon downregulation has not been shown previously for most of the genes listed in Table 1B. Many of these genes are known to participate in cell cycle regulation (e.g., *Mis5*, *Rad51*, *Cdk10*, and *E2F1*) (Gutkind, 2000). Transcript levels for the Bcl-2 antiapoptotic protein were markedly decreased (10-fold), but the expression of genes with proapoptotic function, such as *s-myc* (Asai et al., 1994) and *Ste20-like* protein (Graves et al., 1998), was also reduced following IFN-β treatment. In addition, other genes having no apparent effect on cell cycle or survival exhibited reduced expression (e.g., *tubulin*, *glucoronidase*, and *CD21*). Since this profile of reduced gene expression occurred prior to overt signs of apoptosis, it likely reflects an intermediate phase in the apoptotic program.

Cell Cycle Arrest Precedes Apoptosis in the Pro-B Cell Response to Type I Interferons

When the cell cycle status of the Scid7 cells was examined before and after interferon treatment, the IFN-α/β-treated cells exhibited an obvious G_0-G_1 arrest and a decrease in frequency of cells in the later cell cycle stages by 16 hr (Figure 1A). Apoptosis was manifested at 24 hr by the presence of a large subpopulation of cells with subdiploid amounts of DNA. The reduction in expression of cell cycle-related genes seen in the gene profile analysis is thus mirrored by cell cycle arrest that precedes apoptosis. Although lower transcript levels of the antiapoptotic *bcl-2* gene were evident at the 16 hr time point (Table 1B), a reduction in Bcl-2 protein levels was not evident until the 24 hr sampling interval (Figure 1B). Since reduced levels of Bcl-2 accompanied the onset of apoptosis induced by IFN-α/β, the Bcl-2 reduction could at least in part be attributable to protein degradation as a consequence of the activation of caspases or other proteolytic enzymes. Caspase activation is a

Table 1. *Scid7* Genes Regulated by Interferon-β Treatment

A. *Scid7* Genes Upregulated by Interferon-β Treatment

Gene Name	Gene Description	ISG	Control	Fold Increase
p540ASL	oligoadenylate synthetase-like protein		*	83.9
GARG-16/IFI56	human glucocorticoid-attenuated regulated gene	#	*	68.3
ISG-15/UCRP	ubiquitin cross-reactive protein	#	*	60.9
Mx	antiviral state against the influenza virus	#	*	53.1
UBP43	ubiquitin-specific protease			45.6
TYMK/CDC8	thymidylate kinase homolog		*	41.6
IIGP	IFN-inducible GTPase	#	*	40.7
Irf-7	interferon regulatory factor 7	#	*	37.7
BEST5	rat Best5 protein	#		36.6
Ly6A/E	lymphocyte differentiation antigen	#		31.0
TREX1	3′-5′ exonuclease		*	30.4
Ifi204	interferon-activated gene 204	#	*	29.9
GARG-39/IFI54	glucocorticoid-attenuated response gene 39	#		29.5
Mag-1/GBP-1	IFN gamma inducible protein	#	*	27.6
Stat-1	signal transducer and activator of transcription-1	#		25.9
FLN29	human TRAF interacting Zn finger protein		*	24.8
Ly-6C	Ly-6c		*	24.6
Chop10/GADD153	CHOP-10	#	*	24.4
Ifi202b	interferon-activated gene 202	#	*	23.6
GTP2/Mg21	interferon-induced GTP binding protein	#		23.5
Mg11	interferon-induced GTP binding protein	#	*	22.8
F_cgR	IgG receptor (beta-Fc-gamma-RII)	#	*	19.9
Evi-2	ectotropic viral integration site-2		*	18.4
⟶Daxx	Fas binding protein			17.7
IGTP	GTPase			16.7
L35A	60S ribosomal protein			16.0
GBP-2	guanylate nucleotide binding protein 2			15.8

B. *Scid7* Genes Downregulated by Interferon-β Treatment

Gene Name	Gene Description	Fold Decrease
MIS5	MCM protein. DNA replication in cell cycle	16.3
nm23-M4	nucleoside diphosphate kinase	12.4
GGA2	human ADP-ribosylation factor binding protein	11.2
AES-1(Grg)	aminoterminal-enhancer of split. Enhancer of split Groucho	10.4
FKBP51	FK506 binding protein 51kd. Calcineurin inhibitor. Immunophilin	10.2
Bcl-2	inhibits apoptosis	9.9
ERG2	IFN and steroid inducible protein. Embryo implantation	9.3
PoID2	DNA polymerase delta small subunit	8.3
DNA ligase I	DNA metabolism	7.8
dUTPase	converts dUTP into dUMP+ Ppi	7.6
e-IF4gamma	human translation initiation factor 4G	7.5
LTB	lymphotoxin β	7.3
s-myc	*myc* gene with transactivating activity. Apoptotic inducer	7.2
Rad51	mitotic and meiotic recombination and DNA repair	7.0
SCD	stearoyl-CoA desaturase	6.9
CDK10	human cyclin-dependent kinase-related protein 10	6.5
GTPAAP	GTPase activating protein	6.3
β-tubulin	cytoskeleton	6.2
IF1	protein tyrosine phosphatase	6.1
GUS	β-glucoronidase	5.8
Phb	prohibitin. Intracellular inhibitor of proliferation	5.8
CD21	complement receptor 2	5.6
E2F1	critical determinant for the G1/S transition in the cell cycle	5.6
Cdr2	cerebellar degeneration-related autoantigen-2	5.6
SR-BI	scavenger receptor class B type I. Lipoprotein receptor	5.5
Bub1b	mitotic checkpoint protein kinase	5.2
Hspbp	rat heat shock 70 binding protein	5.2
Ste 20-like	serine-threonine kinases. Role in apoptosis	5.1
MG-160	Golgi apparatus sialoglycoprotein	5.0
ATP-citrate lyase	rat lipogenic enzyme	5.0

Genes ordered according to differences in expression between treated and untreated cells. A # mark in the interferon inducible gene (ISG) column indicates that IFNs have been found to regulate the expression of this gene in previous studies. An asterisk (*) in the control column means that the expression of this gene could not be detected in the untreated sample, and a value was entered by software convention; the actual fold change in this gene therefore may be underestimated. *Daxx* is indicated by an arrow.

general feature in most apoptotic programs, although its importance may vary according to the model of apoptosis (Green, 2000). When the Scid7 pro-B cells were pretreated with Z-VAD-FMK, a cell-permeable generic caspase inhibitor (Hara et al., 1997), Bcl-2 reduction was still evident at 24 hr (Figure 1B), and there was no effect

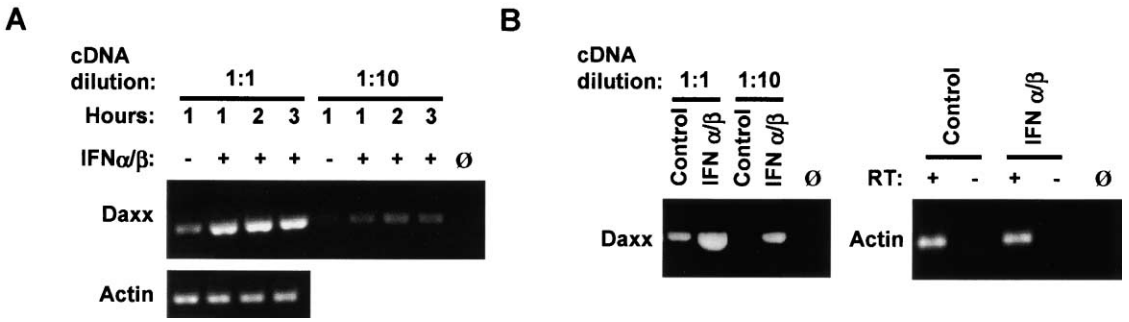


Figure 2. Analysis of the Interferon-α/β Effect on Daxx Expression in Scid7 and *Stat1*$^{-/-}$ Bone Marrow Cells

(A) *Daxx* and β-*actin* transcripts were analyzed by RT-PCR in Scid7 cells treated with IFN-α/β for different time intervals. PCR products in agarose gels were revealed by ethidium bromide staining.

(B) Bone marrow cells from *Stat1*$^{-/-}$ mice were examined before and 16 hr after treatment with IFN-α/β as indicated above. PCR results on control samples not treated with reverse transcriptase (RT) or with no template (Ø) are also shown.

on the interferon-induced cell death (Figure 1C). This finding suggests that caspase activation is not essential for the interferon-induced apoptosis of B lineage progenitors.

Interferon Induction of *Daxx* Expression, a STAT1-Independent Effect

In a search for additional clues to the mechanism of this mode of apoptosis, we concentrated first on Scid7 genes showing increased expression following type I interferon treatment (Table 1A). Since an important goal of these studies was to identify a STAT1-independent pathway of apoptosis, our attention was drawn to highly expressed genes (*Isg20*, *Sp100*, and *Daxx*) known to be components of nuclear bodies, because these structures have been implicated in apoptosis (Hess and Korsmeyer, 1998). Moreover, the overexpression of *Daxx* has been associated with an enhancement of Fas-mediated cell death by apoptosis (Yang et al., 1997).

Because *Daxx* had not been previously recognized as an interferon-inducible gene, we first conducted experiments to confirm the DNA microarray analysis. RT-PCR assessment of the *Daxx* transcript levels in Scid7 cells treated with IFN-α/β confirmed the increased expression of *Daxx* at 16 hr. When this analysis was repeated at earlier time points, the interferon induction of *Daxx* transcription was evident within the first hour of treatment and persisted beyond 16 hr (Figure 2A; Table 1; data not shown). Similar results were obtained when the Scid7 cells were pretreated with the protein synthesis inhibitor cyclohexamide. Although cyclohexamide treatment itself can induce apoptosis, *Daxx* levels were unaffected by 10 hr of cyclohexamide treatment in these experiments (data not shown). These findings indicate that *Daxx* is an early interferon-inducible gene whose upregulation by interferon does not require new protein synthesis, thereby inferring direct signaling via the type I interferon receptor. Since we also sought a signaling pathway that could function in the absence of STAT1, we examined whether or not *Daxx* transcription could be induced by interferons in STAT1-deficient mice. When bone marrow cells from these mice were examined after 16 hr of treatment with IFN-α/β, increased levels of *Daxx* transcripts were detected (Figure 2B), thus indicating that *Daxx* expression is interferon inducible via a STAT1-independent signaling pathway.

Alteration of Daxx Protein Expression by IFN-α/β Treatment

In order to evaluate changes in the level and intracellular localization of Daxx protein, Scid7 cells were examined before and after interferon treatment by immunofluorescence confocal microscopy with a polyclonal anti-Daxx antisera. A diffuse cytoplasmic distribution of Daxx was observed for most of the untreated cells, although some also contained Daxx in a punctate nuclear distribution pattern (Figure 3A). IFN-α/β treatment induced an obvious change in Daxx expression, whereby virtually all of the treated cells exhibited an increase of Daxx within the nuclear bodies (Figure 3A). Analysis of intracellular Daxx protein levels in these experiments indicated increased expression within 8 hr after the initiation of the interferon stimulation (Figure 3B), that is, before overt cell cycle arrest (data not shown). The interferon-induced increase in *Daxx* transcription was thus accompanied by a prompt increase in Daxx protein and its localization in nuclear bodies. This alteration in Daxx expression appears to be a characteristic interferon effect, as it was not seen in Scid7 cells deprived of the IL-7 growth stimulus or following dexamethasone treatment (Figure 3A), although Scid7 apoptosis is induced under both conditions (Wang et al., 1995; data not shown).

IFN-α/β Induction of Daxx Expression in Primary Pro-B Cells

Having observed that type I interferons can induce Daxx upregulation and nuclear translocation in the Scid7 pro-B cell line, we examined the cellular distribution of Daxx and the effects of type I interferon on its expression in primary B cell progenitors. B lineage subpopulations in the bone marrow were analyzed in the initial experiments. Three subpopulations were characterized by using the B220 and CD43 markers (Hardy et al., 1991): B220^{+}CD43^{+} (including most B cell progenitors), B220^{+}CD43^{-} (pre-B and immature B cells), and B220^{high}CD43^{-} (mature B cells). Daxx-containing nuclear spots were observed in cells belonging to all three subpopulations, but cells within the pro-B cell subpopulation (B220^{+}CD43^{+}) contained more Daxx-positive nuclear bodies than cells in the other two subpopulations (data not shown). Since the in vivo Daxx distribution pattern may be influenced by type I interferons produced

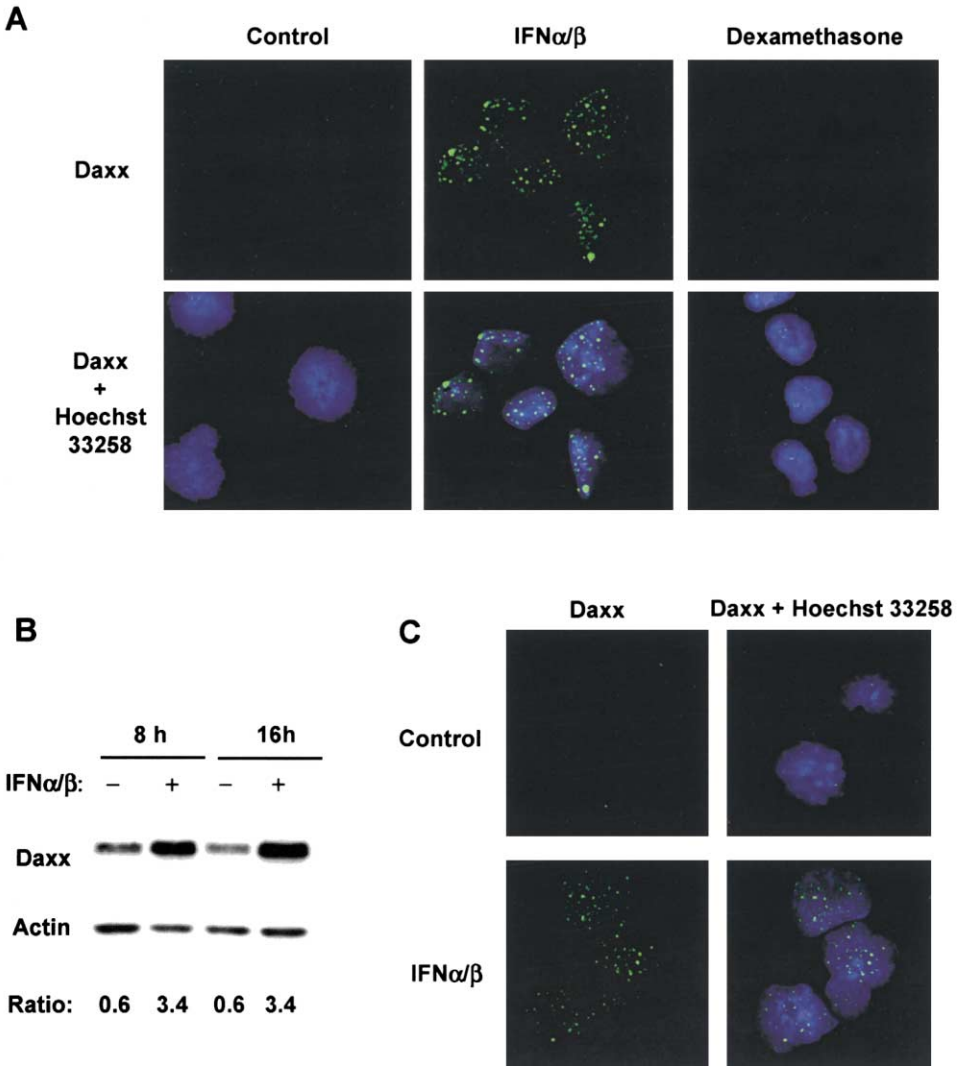


Figure 3. Expression and Localization of Daxx Protein in B Cell Progenitors

(A) Immunofluorescence analysis of Daxx expression by Scid7 pro-B cells before and 16 hr after treatment with IFN-α/β or dexamethasone. Staining with Daxx antibody is green, and nuclear staining with the fluorescent blue Hoechst 33258 dye is visualized by confocal microscopy.
(B) Western blot analysis of Daxx and actin levels in Scid7 cells treated or untreated with interferon for 8 and 16 hr.
(C) Analysis of interferon-α/β induction of Daxx expression in B lymphocyte progenitors derived from fetal liver. $B220^+CD43^+$ progenitor B cells isolated by sorting from short-term fetal liver cell cultures were recultured with or without IFN-α/β in media containing IL-7 before immunofluorescence analysis of Daxx expression by confocal microscopy.

by bone marrow macrophages (Wang et al., 1995), we examined whether or not type I interferons could induce an increase in Daxx expression by primary B lineage cells in an ex vivo environment. For these studies, a model of B lymphopoiesis was employed, in which bone marrow or fetal liver progenitors were grown on IL-7-transfected fibroblasts to promote efficient growth and differentiation of B cell progenitors (Borzillo et al., 1992; Lin et al., 1998). $B220^-$ lymphoid progenitors from the liver of normal 15 day embryos were placed in this ex vivo environment for 4 days, after which their $B220^+CD43^+IgM^-$ pro-B cell progeny were purified by fluorescence-activated cell sorting before treatment with IFN-α/β for 16 hr in IL-7 containing media. Immunofluorescence analysis of the interferon-treated cells indicated increased levels of the Daxx protein that was concentrated primarily within the nuclear bodies (Figure 3C). Daxx upregulation and nuclear body translocation is thus induced by type I interferons in primary B cell progenitors as well as in the Scid7 pro-B cell line.

Fas Expression Is Not Required for IFN-α/β-Mediated Inhibition of B Lymphopoiesis

Daxx was identified initially as a Fas binding protein in an apoptotic signaling pathway differing from that involving activation of the death-inducing signaling complex (Fas, FADD, procaspase-8) leading to caspase-8 activation (Yang et al., 1997). Daxx overexpression enhances Fas-induced apoptosis, although it does not induce apoptosis per se and does not augment other apoptotic stimuli (Yang et al., 1997; Torii et al., 1999). In an immunofluorescence analysis, we found that treatment with type I interferons did not upregulate Fas in

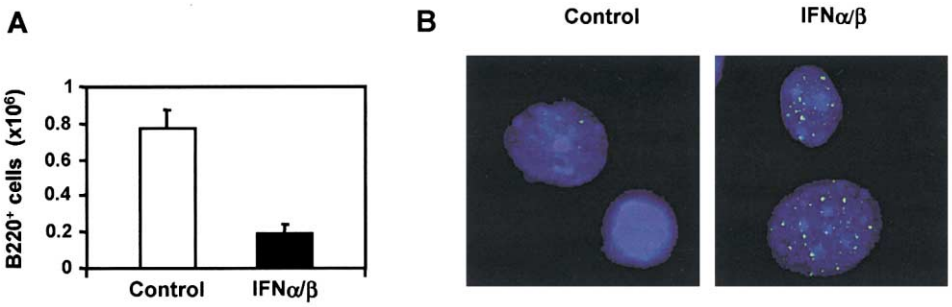


Figure 4. Interferon-α/β Effects on B Lymphocyte Progenitors from Fas-Deficient Mice

(A) Analysis of interferon-induced growth inhibition. Fetal liver cells from *lpr/lpr* mice were cultured for 4 days in an ex vivo B lymphopoiesis system in the presence or absence of IFN-α/β. Viable cells, identified by trypan blue exclusion, were then counted.

(B) Immunofluorescence analysis of Daxx distribution in $B220^{+}CD43^{+}$ progenitor B lineage cells derived from *lpr/lpr* mice and cultured with or without interferon as described in (A).

primary B cell progenitors nor in the Scid7 pro-B cells, both of which express Fas at very low levels (Mandik et al., 1995; data not shown). However, to exclude the possibility of a Fas effect in the apoptotic pathway triggered by type I interferon, we also examined the IFN-α/β response by B cell progenitors from *lpr/lpr* mice that lack a functional Fas receptor (Watanabe-Fukunaga et al., 1992). When *lpr/lpr* fetal liver progenitors were monitored in the ex vivo lymphopoiesis model described above, their growth and survival was effectively inhibited by IFN-α/β treatment (Figure 4A) IFN-α/β also induced Daxx upregulation and nuclear body localization in the Fas-deficient pro-B cells (Figure 4B).

Daxx Expression Is Essential for the Inhibitory Effect of Type I Interferons

Given the increased level of Daxx expression observed in the nuclear bodies of progenitor B cells treated with interferon, we wished to determine whether or not Daxx is required for the ensuing apoptosis. Since the Daxx null mutant is embryonically lethal (Michaelson et al., 1999) and our DNA microarray, RT-PCR, and immunofluorescence analyses indicate that Daxx is expressed in B cell progenitors, even without treatment, we sought to transiently reduce the expression of Daxx prior to interferon treatment by the use of antisense (AS) Daxx oligonucleotides. The antisense oligos pair with nascent RNA, resulting in degradation by cellular RNases, as occurs in viral infections (Praseuth et al., 1999). In these experiments, in which sense oligos, "scrambled" nonsense oligos, and water were used as controls, Scid7 cells preloaded with the different oligos grew well in the absence of IFN-α/β treatment, and their growth was inhibited by IFN-α/β treatment. In contrast, when the cells were pretreated with the Daxx antisense oligos, the IFN-α/β growth inhibitory effect was abolished (Figure 5A). As anticipated, the antisense oligo also inhibited the interferon-induced increase in Daxx protein expression (Figure 5B). Immunofluorescence analysis of these cells confirmed that treatment with the Daxx antisense oligos inhibited the interferon-mediated Daxx upregulation and the nuclear body localization of this protein, whereas treatment with the control oligos or water had no effect in these experiments (Figure 5C).

Since, in this model system as in other apoptotic models, cell cycle arrest precedes the apoptosis induced by interferons (Figure 1A) (Gutkind, 2000), we wished to determine whether the antisense Daxx oligonucleotide affected both components of the interferon response. In these experiments, the Scid7 cells were preloaded with the Daxx antisense or control oligos, and the cell cycle status was analyzed before and after treatment with interferon for 16 and 24 hr. In addition to the inhibition of Daxx protein expression, the Daxx antisense oligo also rescued the interferon-treated pro-B cells from both cell cycle arrest and the ensuing apoptosis (Figure 6).

Discussion

This analysis indicates an important role for the Daxx protein in the type I interferon-mediated inhibition of B lymphopoiesis. Enhanced Daxx expression was found to be essential in this Fas-independent apoptotic process, although its role here clearly differs from that originally described, as a consequence of its interaction with the Fas death domain (Yang et al., 1997). The present results indicate that this interferon-triggered pathway of apoptosis involves translocation of Daxx to nuclear bodies and the related suppression of cell cycle-related genes.

Nuclear bodies, also called promyelocytic leukemia (PML) oncogenic domains (PODs) (Dyck et al., 1994), Kr bodies (del Mazo et al., 1987), or nuclear dot 10s (ND10) (Ascoli eand Maul, 1991), can be detected in all types of cells. Although their protein composition, dynamics, and functions are still incompletely resolved, nearly 20 nuclear body-associated proteins have been identified. Nuclear body numbers and composition change in response to variations in cell cycle, viral infection, and other conditions, indicating the functional complexity of these cellular components (Hess and Korsmeyer, 1998; Everett et al., 1999). Much of the current interest in nuclear bodies has been stimulated by the analysis of acute promyelocytic leukemia (APL), a myeloid malignancy that has been causally related to a disturbance of nuclear bodies due to the presence of the PML-RARα fusion protein created by translocation of the *Pml* and retinoic acid receptor (*Rar*) genes (Kakizuka et al., 1991). In these leukemic cells, the nuclear body components are delocalized into smaller particulate structures called microspeckles. When the APL cells are treated with all-*trans*-retinoic acid, the therapeutic agent used for these patients, the PML-RARα fusion protein is degraded, Pml

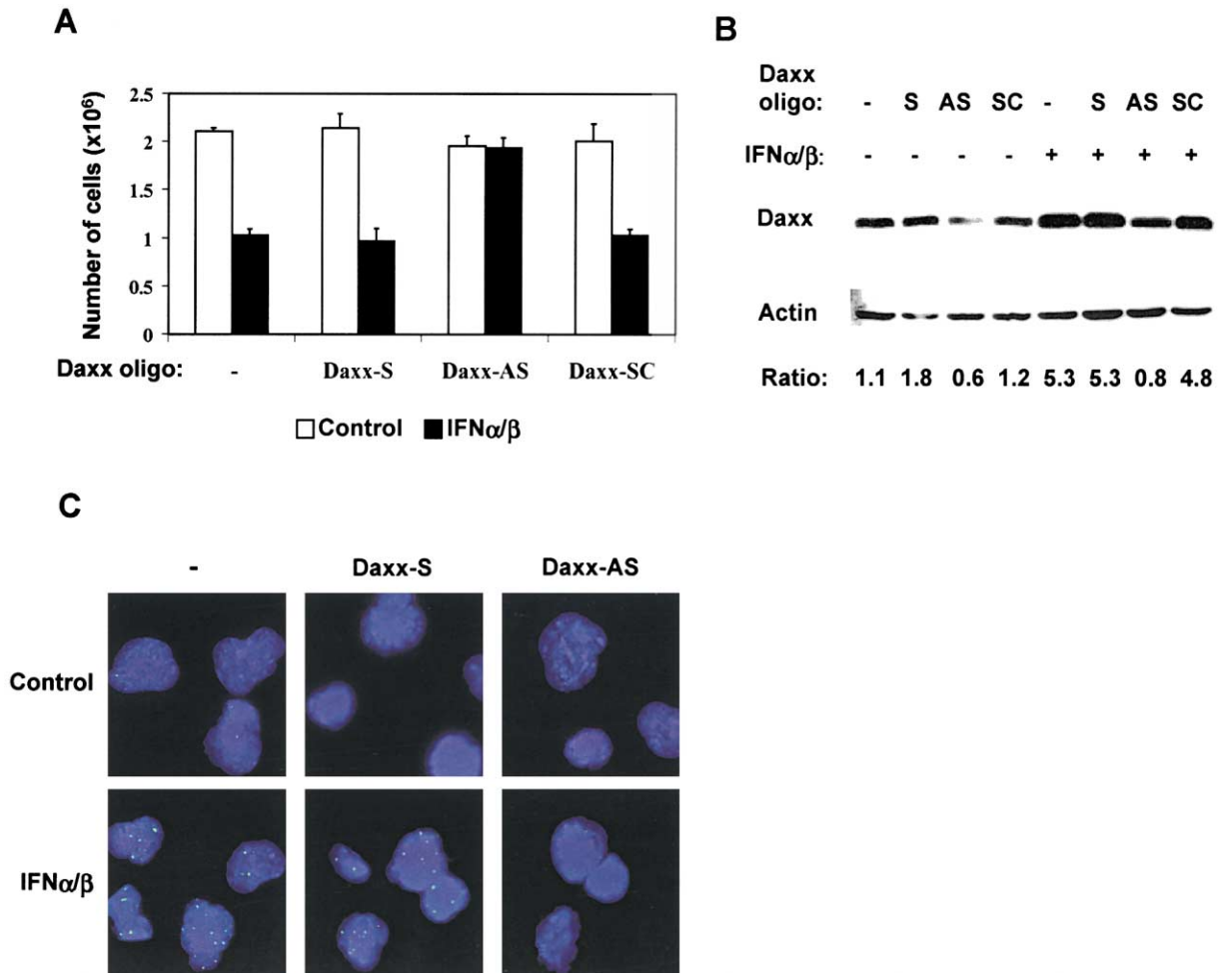


Figure 5. Effect of Daxx Antisense Treatment on the Interferon-α/β Response of Scid7 Cells

(A) Scid7 cells preloaded for 8 hr with Daxx sense (Daxx-S), antisense (Daxx-AS), or scrambled (Daxx-SC) oligonucleotides or with water (H_2O) were cultured in fresh media with or without IFN-α/β for 24 additional hr before harvesting and enumeration of viable cells.

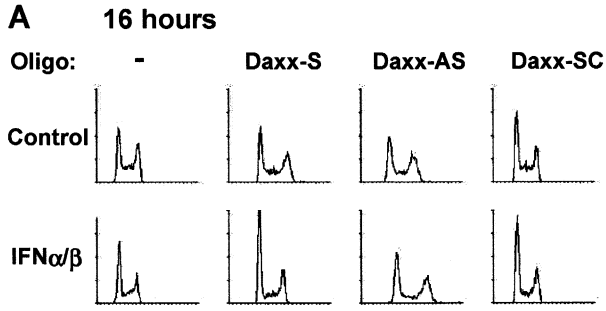
(B) Scid7 cells were cultured for 16 hr as in (A) before determination of intracellular Daxx and actin levels by Western blot analysis as indicated in Figure 3.

(C) Scid7 cells preloaded with the oligos or water were cultured for 16 hr with or without IFN-α/β before immunofluorescence analysis of Daxx expression by confocal microscopy.

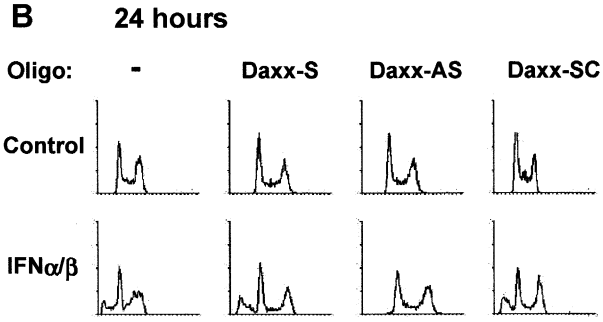
relocalizes into the nuclear bodies, and the leukemic cells undergo differentiation (Wang et al., 1998a). A related finding in Pml-deficient mice is that their myeloid progenitors are resistant to the apoptotic effects of a variety of stimuli, including UV light, interferons, ceramide, and Fas ligation (Wang et al., 1998b).

Isg20, Sp100, and Daxx are other protein components that have been used to define nuclear bodies in humans, and the genes for all of these proteins have now been shown to be interferon inducible (Guldner et al., 1992; Grotzinger et al., 1996; Gongora et al., 1997, 2000b; see Results). High levels of transcripts for some of these genes were detected in our DNA microarray analysis of the interferon response prior to overt signs of apoptosis. *Daxx* and *Isg20* genes were among the genes for which we observed the highest levels of interferon-induced expression. A murine EST with a short region of homology to human *Sp100* was also greatly increased. Conversely, *Pml* expression, of particular interest in view of its demonstrated role in apoptosis, was minimally enhanced by the interferon treatment. Moreover, interferon-mediated regulation of the *Pml* gene has been shown to require STAT1 expression (Stadler et al., 1995). Changes in Pml expression are therefore unlikely to account for the STAT1-independent apoptotic response of early B progenitors to the type I interferons, although it could be postulated that basal levels of this protein are required.

In our analysis of B cell progenitors, we found that *Daxx* expression is promptly induced by interferon treatment, independently of new protein synthesis or the presence of STAT1. The analysis of *lpr/lpr* mice, which lack Fas expression, also indicated that Fas is not required for Daxx induction and the type I interferon-mediated apoptosis of B cell progenitors. In the progenitor B cells treated with interferon, an accumulation of Daxx was observed within the nuclear bodies prior to overt apoptosis. When mouse splenocytes were treated with Concanavalin A in another recent study, most of the B cells died after 3 days by a mechanism involving nuclear



IFNα/β	Oligo	< 2 n	G_0-G_1	S	G_2-M
-	-	0.4	39.1	33.8	26.6
-	Daxx-S	0.2	35.0	33.8	27.2
-	Daxx-AS	0.5	37.8	28.4	30.1
-	Daxx-SC	0.5	42.5	32.3	20.3
+	-	0.9	49.8	27.0	22.4
+	Daxx-S	0.2	54.8	23.6	19.6
+	Daxx-AS	0.4	40.9	24.6	31.4
+	Daxx-SC	0.3	53.8	23.3	21.7



IFNα/β	Oligo	< 2 n	G_0-G_1	S	G_2-M
-	-	0.3	32.4	30.7	33.9
-	Daxx-S	0.2	39.5	27.5	28.6
-	Daxx-AS	0.1	35.9	27.6	32.3
-	Daxx-SC	0.2	47.0	28.0	23.0
+	-	21.0	25.4	18.6	34.5
+	Daxx-S	21.8	35.9	13.2	28.7
+	Daxx-AS	0.4	36.5	23.0	39.3
+	Daxx-SC	21.2	30.4	15.6	32.5

Figure 6. Influence of Daxx Antisense Treatment on the Antiproliferative and Apoptotic Effects of Interferon

Scid7 pro-B cells were preloaded with oligos for Daxx, as indicated in Figure 5, before treatment with interferon and cell cycle analysis at 16 hr (A) or 24 hr (B). Percentages of cells in the different compartments of the cell cycle are indicated in the tables.

body colocalization of Pml and Daxx (Zhong et al., 2000). Pml, Isg20, Sp100, and other nuclear body-associated proteins could interact with Daxx as participants in the apoptotic program initiated by type I interferons in IL-7-dependent lymphoid progenitors.

Proteins localized within the nuclear bodies have been implicated in the regulation of transcription. Nascent RNA has been reported in these structures (LaMorte et al., 1998), although the failure to demonstrate RNA in the nuclear bodies in a recent study suggests these may not be sites in which transcription occurs (Boisvert et al., 2000). Among the nuclear body components known to be transcriptional regulators, Daxx has been shown to mediate both basal and activated gene repression (Hollenbach et al., 1999; Li et al., 2000a, 2000b). The mechanism for the Daxx-mediated repression is unknown, but its interaction with the histone deacetylase HDAC1 suggests that histone deacetylation may be involved (Li et al., 2000a). Gene repression induced by the physical interaction of Daxx with activating transcriptional factors, such as Pax3 and Ets-1, has been also described (Hollenbach et al., 1999; Li et al., 2000b).

Our DNA microarray analysis of Scid7 cells indicated that the increase in Daxx expression induced by type I interferons temporally correlates with a relatively broad repression of gene expression that precedes the apoptosis of B cell progenitors. Several of the downregulated genes, including *Mis5*, *Rad51*, *Cdk10*, and *E2F1*, are known as cell cycle regulators (Gutkind, 2000), and cell cycle arrest was found to precede apoptosis. The increase in Daxx protein levels occurred even earlier in the interferon-treated progenitor B cells, and the possibility that Daxx is involved in mediating the cell cycle arrest as well as subsequent apoptosis was supported by the finding that pretreatment with antisense oligonucleotides alleviated both the cell cycle arrest and apoptosis in the interferon-stimulated Scid7 cells. In this context, Daxx has recently been described as a repressor of *bcl-2* gene expression (Li et al., 2000b), and a dramatic reduction of *bcl-2* transcription preceded apoptosis in the type I interferon-treated B cell progenitors in our experiments. In addition, we have shown in previous studies that *bcl-2* overexpression in pro-B cells selectively counteracts the apoptotic effect of interferon without significantly altering the growth inhibition (Wang et al., 1995).

Finding that *Pml* overexpression can reverse Daxx-mediated repression of transcription, Li et al. (2000a) proposed that Pml, itself a transcriptional activator, may block the transcriptional repression by binding the Daxx protein and sequestering it within the nuclear body complex. This attractive hypothesis does not easily explain the viability of Pml-deficient mice (Wang et al., 1998a) or the fact that Daxx-deficient mice die early in embryogenesis (Michaelson et al., 1999). It is also uncertain whether this hypothesis could account for the type I interferon effect on B cell progenitors, since the growth inhibition and apoptosis were associated with prominent nuclear body localization of Daxx. Although interferon may induce enhanced expression of both *Daxx* and *Pml* genes, we observed that *Daxx* expression was enhanced to a much greater extent than *Pml* expression (17.7-fold versus 1.9-fold) in the B cell progenitors. Given the complexity of nuclear body protein composition, a delicate balance may exist between interacting nuclear body proteins with either repressor or activating functions, so that the selective perturbation of these regulatory elements could dictate life or death of the cell.

Variation in the overall composition of the nuclear body protein complex as a function of cell type and differentiation status may also contribute to the equilibrium, thereby accounting for the differences observed in cellular responses to interferon. As an example, whereas type I interferons induce apoptosis in pro-B cells (Wang et al., 1995; Gongora et al., 2000a; see present Results), they inhibit the B cell receptor-mediated apoptosis of mature B cells (Su and David, 1999).

In conclusion, these experiments reveal an unanticipated role for Daxx in type I interferon-mediated inhibition of IL-7-dependent B lineage progenitors. Since type I interferons are equally inhibitory for intrathymic T cell progenitors (Su et al., 1997; Lin et al., 1998), this STAT1- and Fas-independent pathway of apoptosis may be involved in the interferon-mediated inhibition of both T and B lymphopoiesis.

Experimental Procedures

Mice, Cell Cultures, and Modifying Reagents

Stat1-deficient mice of the 129 Sv/Ev background (Meraz et al., 1996) were obtained from Dr. Robert Schreiber (Washington University, St. Louis, MO), and the 129 Sv/Ev mice and B6.MRL^{lpr} mice were obtained from Jackson Laboratories. The Scid7 cell line derived from a Scid mouse was maintained as described (Ogawa et al., 1989). In an ex vivo model of B lymphopoiesis, B cell progenitors from the liver of 15-day-old embryos were cultured with IL-7-transfected NIH 3T3 fibroblasts (Borzillo et al., 1992) at a concentration of 10^6 cells/ml in RPMI 1640 medium with 5% FCS, L-glutamine, penicillin/streptomycin, and 50 μM β-ME. IFN-β, an IFN-α/β combination isolated from virus-infected cultures, and control supernatants from mock-infected cultures were purchased from Access Biomedical (San Diego, CA). Cyclohexamide was obtained from Calbiochem (La Jolla, CA) and dexamethasone from Biomol (Plymouth Meeting, PA). The caspase inhibitor Z-VAD-FMK was purchased from Sigma (Saint Louis, MO).

Analysis of Gene Expression by DNA Microarrays

Scid7 cells were processed before and after treatment with IFN-β to obtain poly(A)$^+$ RNA by using the FastTrack 2.0 Kit (Invitrogen, Carlsbad, CA). Methods for cRNA synthesis from poly(A)$^+$ and additional procedures leading to the hybridization and scanning of GeneChip arrays were conducted as recommended by the manufacturer (Affymetrix, Santa Clara, CA). Poly(A)$^+$ RNA (2 μg) was used for the analysis with two sets of murine GeneChips, Mu19K and Mu11K. Mu19K contains probes interrogating 19,000 murine ESTs from TIGR (1.0 Beta), and Mu11k contains approximately 11,000 genes and ESTs from Unigene (Build 4) and 800 EST clusters from TIGR (1.0 Beta); approximately 20%–25% redundancy exists between the two sets. A mixture of four control cRNAs for bacterial and phage genes was included in the hybridization solution to serve as a reference for comparison of hybridization efficiency between arrays and for the relative quantification of transcript levels. The GeneChip Analysis Suite 3.3 package (Affymetrix) was used to analyze the scanned images and to process the data. Accurate orientation for scanning of the array was achieved by addition of a biotinylated oligonucleotide (B2) that hybridized to unique features in the four corners and center of each chip. Comparable background autofluorescence values were found for the arrays corresponding to control (658 ± 49) and IFN-β-treated (645 ± 33) cells (mean fluorescence intensity ± SD). Additionally, the ratios of fluorescence intensities for the 3′ and 5′ parts of the *Gapdh* gene were within the normal range of variation: control (0.85 ± 0.02) and IFN-β-treated (0.82 ± 0.04) cell samples. Similar results were found in the case of the β-*actin* gene for control (0.96 ± 0.02) and IFN-β-treated (1.27 ± 0.03) cell samples.

Gene identification for some ESTs was accomplished by BLAST search of EST sequences against existing databases, and, in other cases, murine ESTs were tentatively identified as homologs of mammalian genes by their relatively high sequence homology (>80% over a >400 bp segment).

RT-PCR Analysis

RNA isolated from freshly harvested cells using the TRI Reagent (Molecular Research Center, OH) was dissolved in DEPC-treated water. The samples were divided into two parts: one treated with reverse transcriptase and the other untreated. First-strand cDNA synthesis from RNA was carried out using poly(dT) and reverse transcriptase enzyme in the SuperScript Preamplification System (GIBCO, Rockville, MD), following protocols supplied by the manufacturer. For evaluation of Daxx expression, forward 5′- CCCATGG CCACCGATGACAGCAT-3′ and reverse 5′-AGGGTTAGGGCCC GACGCCTCACT-3′ primers were used. Expression of the β-*actin* gene was examined by using forward 5′-TAGACTTCGAGCAGGAG GAGATG-3′ and reverse 5′-CGTACTCCTGCTTGCTGATCCA-3′ primers. The PCR mixture was denatured in a thermal cycler at 94°C for 3 min, and then 25 cycles were performed, each consisting of a denaturation step at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. Dilutions of the cDNA templates were performed to compare transcript levels.

Monoclonal Antibodies, Cell Sorting, Cell Cycle Analysis, and Apoptosis Detection

FITC-labeled monoclonal antibodies to CD43, PE-labeled antibodies to CD45R (B220), and Cy-Chrome-labeled anti-μ heavy chain antibodies, and an anti-Fas monoclonal antibody were obtained from PharMingen (San Diego, CA). Cell suspensions incubated on ice for 25 min with fluorochrome-labeled antibodies were washed with phosphate-buffered saline containing 3% FCS and 0.02% NaN_3 before sorting in a Moflow flow cytometer (Cytomation, Ft. Collins, CO). For cell cycle analysis, cells suspended in ice-cold ethanol overnight were pelleted by centrifugation and treated with RNase (Sigma) for 20 min at 37°C before incubation in the dark at room temperature for 15 min in a solution containing propidium iodide at 25 μg/ml and analyzed for DNA binding of propidium iodide with a FACScalibur flow cytometer (Becton Dickinson). Apoptotic cells were identified by their subdiploid DNA content or by Annexin V binding and exclusion of propidium iodide as described by Vermes et al. (1995).

Western Blot Protein Analysis

Cells (5×10^7) were lysed in 500 μl of lysis buffer [1% NP-40 in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), containing 5 mM EDTA, 20 mM iodoacetamide, 0.1% sodium azide, 20 mM ε-amino-caproic acid, antipain (2 μg/ml), leupeptin (1 μg/ml), aprotinin (2 μg/ml), chymostatin (2.5 μg/ml), and pepstain (1 μg/ml)]. Cell lysate proteins were separated by SDS-PAGE before transfer by electroblotting onto nitrocellulose membranes that were incubated sequentially with rabbit anti-Bcl-2 antibodies (Santa Cruz Biotechnology, CA) and horseradish peroxidase-labeled goat antibodies to rabbit Ig (Santa Cruz Biotechnology). The Bcl-2 antigen was visualized by enhanced chemiluminescence (Amersham Life Science, Airlinton Heights, IL). To confirm that the lanes were loaded with equivalent amounts of protein, the filters were stripped following standard protocols and then incubated with goat anti-actin antibodies and horseradish peroxidase-labeled donkey antibodies to goat Ig (Santa Cruz Biotechnology) before chemiluminescence detection. The relative intensities of the bands in X-ray films were determined using the Fluour-S MultImager system (Bio-Rad, Hercules, CA) and the values expressed as the ratio of Daxx protein versus actin in cell lysates.

Immunocytochemistry

Cells centrifuged onto glass slides were fixed in cold methanol (−20°C) for 20 min and were washed extensively in PBS before blocking with 10% normal goat serum and incubation for 1 hr either with rabbit polyclonal anti-Daxx antibodies (Santa Cruz Biotechnology) or with nonimmune rabbit serum as a negative control. The slides were then incubated for 45 min with an Alexa fluor 488-conjugated goat anti-rabbit IgG (H + L) antibody. To reveal cell nuclei, slides were rinsed in PBS containing Hoechst 33258 at 20 μg/ml for 4 min before washing in PBS. Finally, slides were mounted with a coverslip in Fluoromount G (Southern Biotechnology, AL) and stored at −20°C before examination by confocal microscopy.

Antisense Oligonucleotides

Daxx antisense (Daxx-AS), sense (Daxx-S), and scrambled (Daxx-SC) phosphothioate oligonucleotides (Research Genetics Inc., Huntsville, AL) were dissolved in water. Daxx-AS (5′-CGGTG GCCATGGGGGTTC-3′), Daxx-S (5′-AATTTGAACCCCCATGGC-3′), and Daxx-SC (5′-CGGGTGCCTGTGGAGGTC-3′) were designed to contain the transcription initiation sequence. The Daxx-SC oligo retains the 5′ CpG sequence, and the order of the other nucleotides was randomly altered; this oligo was designed to exclude the possible proliferative effect of the CpG dinucleotide (Krieg et al., 1995). Cells were preloaded with the oligos at 280 μg/ml for 8 hr before dilution in fresh media and were treatment with interferon for 16 hr. Viable cells were identified by trypan blue (0.4%) exclusion and were counted in a hematocytometer by light microscopy.

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